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TITLE: Production of a Recombinant E. coli Expressed Malarial Vaccine from the C-Terminal Fragment of Plasmodium Falciparum 3D7 Merozoite Surface Protein-1

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13. ABSTRACT *(Maximum 200 words)*
P. falciparum Merozoite Surface Protein-1 (MSP-1) is a leading erythrocytic stage vaccine candidate. Following secondary proteolytic processing, it may play a role in binding and/or invasion of erythrocytes. The secondary processing gives a 33kD and 19kD fragment. The MSP1-19 is a target for parasite inhibitory antibodies and protective immunity. However, it apppears to lack T-helper epitopes. Since antibody is likely the effector mechanism induced by MSP1-19, it is important to insure that recombinant vaccines based on this antigen be folded correctly and contain T-helper epitopes that will enhance induction of humoral responses. We have developed recombinant MSP1-42 molecules that are structurally correct based on positive reactivities with conformation-dependent mAbs. These proteins have been produced to greater than 95% purity and exceed FDA endotoxin standards. Immunogenicity studies in mice and Rhesus monkeys reveal that good antibody responses are induced to MSP1-42 when these animals are immunized with the MSP1-42 plus SBAS2 adjuvant.

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INTRODUCTION

The objective of this work was to produce cGMP-grade; properly folded *E. coli* expressed MSP-1_{42} antigens for human Phase I clinical trials. *In vitro* studies have shown that protective epitopes on MSP-1 are present on the C-terminal MSP-1 cleavage product, MSP-1_{42} (Egan, 1999). However, the C-terminal MSP-1_{42} contains complex tertiary structure through 6 disulfide bridges. Correct presentation of protective epitopes on MSP-1_{42} is dependent on proper disulfide bridge formation. Plasmids containing the MSP-1_{42} gene sequence were used to transform *E. coli* and express recombinant MSP-1_{42} (either the 3D7 or FVO strain). Purification processes to purify the recombinant MSP-1_{42} included affinity purification through Histidine binding through metal chelating resins and ion exchange chromatography. Purified MSP-1_{42} was characterized for correct structure using disulfide-dependent mAbs specific for epitopes on either MSP-1_{19} or MSP-1_{33}. MSP-1_{42} (3D7) reconstituted with SBAS2 adjuvant was used to immunize mice and Rhesus monkeys to study the induction of MSP-1 specific antibodies.

Background Review

The mosquito-born parasite *Plasmodium falciparum* is the leading cause of clinical malaria. Greater than 300 million cases of clinical malaria are reported yearly. Approaches to control malaria include mosquito vector-control strategies and chemotherapeutics, as well as development of recombinant antigens for use as vaccines to induce specific immunity against native malarial antigens. Several vaccine candidates have been identified from each of the parasite's

developmental stages. Vaccines that are derived from malaria erythrocytic-stage antigens are of special interest because erythrocytic stages are the only confirmed targets of natural immunity among individuals from malaria endemic regions. The development of protective antibodies against malaria antigens is supported by studies in which immunoglobulin from individuals protected from *P. falciparum* infection was passively transferred into Thai patients infected with malaria (Sabchareon, 1991). The level of parasites in these individuals treated with the protective IgG was reduced.

The major merozoite surface protein-1, MSP-1, is the leading erythrocytic stage vaccine candidate [Diggs, 1993]. The objective of erythrocytic stage vaccines is to diminish the level of parasitemia in the bloodstream and thus reduce the severity of disease. MSP-1 has been studied extensively [Holder, 1988, Miller, 1993], however, its role in erythrocyte binding and invasion remains to be determined[Holder and Blackman, 1994]. The 195kDa MSP-1 is processed to several polypeptides having 83, 28-30, 38-45 and 42 kDa during merozoite development. Merozoite-specific antibodies recognize these proteolytic forms [Holder and Freeman, 1984; Lyon, 1986; Holder, 1987]. These fragments remain associated to the merozoite surface through non-covalent interactions [McBride and Heidrich, 1987; Lyon, 1987] and are attached to the merozoite surface through the C-terminal 42 kDa fragment (MSP-1_{42}). Secondary processing is required for invasion and yields a 33-kDa fragment and a 19 kDa C-terminal fragment (MSP-1_{19}) [Blackman, 1991]. Immediately following invasion, MSP-1_{19} is present on ring-forms in the newly invaded erythrocyte [Blackman, 1990].

The evidence for using MSP-1, specifically the C-terminal fragments, MSP-1_{42} and MSP-1_{19}, as components of erythrocytic stage malaria vaccines is extensive. MSP-1_{19}-specific mAbs inhibit *P. falciparum* growth *in vitro,* [Blackman, 1990], and passively protect mice against infection with *P. yoelii,* [Majarian, 1984; Ling, 1994]. Immunization of monkeys with native MSP-1, [Siddiqui, 1987], baculovirus-expressed recombinant MSP-1_{42} [Chang, 1996], or *S. cerevisiae*-secreted MSP-1_{19} (EVE-MSP-1_{19}) from *P. falciparum* [Kumar, 1995], protect against a homologous challenge. Similarly, *E. coli*-expressed MSP-1_{19} from *P. yoelii,* [Holder, 1994; Burns, 1989] protects against a homologous murine challenge. Anti-sera raised against recombinant MSP-1_{42} [Chang, 1992], or MSP-1_{19} [Lyon and Haynes, unpublished] inhibit *P. falciparum* growth *in vitro*. The MSP-1_{19}-specific mAbs that either protect against infection *in vivo* [Burns, 1989], or inhibit parasite growth *in vitro* [Blackman, 1990], are specific for discontinuous epitopes since they do not react with disulfide-reduced MSP-1_{19} [McBride and Heidrich, 1987; Farley and Long, 1995]. MSP-1_{19} folds into a complex disulfide bridged structure (Holder, et al. ref) with homology to epidermal growth factor (EGF) at the level of the cysteines [Blackman, 1991]. Within a span of 100 amino acid, two EGF-like domains contain six cysteine residues that form three disulfide bridges per domain.

Body

The objective of this study was to produce recombinant MSP-1_{42} from *Plasmodium falciparum* 3D7 and FVO strains expressed from *E. coli* that could be tested for safety and immunogenicity in Phase I clinical trials. To meet this

objective, plasmids that expressed soluble MSP-1_{42} were constructed using recombinant DNA technology. The cloned products were tested for soluble expression in *E. coli* BL21 (DE3) hosts. Recombinant MSP-1_{42} proteins were purified to greater than 95% purity under cGMP standards at the Walter Reed Army Institute of Research, Department of Biologics Research, Pilot Production Plant, Forest Glen, MD. Purification processes were developed using *E. coli* paste derived from 300L cGMP fermentation's also produced at the WRAIR, Department of Biologics Research, Pilot Plant, Forest Glen, MD.

Recombinant MSP-1_{42} proteins were immunoreactive against MSP-1_{19} specific monoclonal antibodies, including the inhibitory mAbs that inhibit processing and invasion (12.10, 12.8) and the blocking mAbs, that block inhibitory mAbs activity (2.2, 7.5, 1E1). The MSP-1_{42} (3D7) strain was reactive against MSP-1_{33} mAbs (7F1), a mAb that is 3D7 strain specific.

To study the induction of MSP-1_{42} (3D7) specific antibodies, Balb/C mice were immunized with MSP-1_{42} (3D7) plus SBAS2 adjuvant (SmithKline Beecham Biologics, Belgium). Mice were immunized at 3-week intervals subcutaneously with 0.1, 0.3 or 1.0 μg's of MSP-1_{42} (3D7)/SBAS2. Sera were analyzed by ELISA against yeast-expressed MSP-1_{19} coated plates. Seroconversion rates following the first and second immunization are shown at the 99% confidence interval (Figure 2). At the highest dose, 1.0 μg, we observed 100% seroconversion following one immunization, while at the lowest dose, 0.1 μg, we observed seroconversion only following two immunizations.

These mice were boosted at 3-week intervals for a total of 5 immunizations and sera from these mice were collected and analyzed for induction of MSP-1_{19} specific antibodies following each immunization. Mice immunized with the highest dose, which was 1.0 μg, produced high antibody titers following the 2nd immunization and this titer was not boosted appreciably following further immunization. Mice immunized with the lowest dose, which was 0.1 μg, eventually produced the same antibody titer as mice immunized with the higher doses (Figure 3). We observed good seroconversion rates in mice after one or two immunizations. After five immunizations the mean antibody titers in these mice exceeded 1:80,000 independent of dose.

Immune sera from mice that were immunized 5 times with 1.0 μg MSP-1_{42} (3D7)/SBAS2 were tested by IFA using serum dilutions of 1:1,000 and 1:10,000. At the 1:1,000 dilution, all of the sera were IFA positive. At the 1:10,000 dilution, 70% of the sera were IFA positive (Table 1). These data indicated that mice immunized with MSP-1_{42} (3D7)/SBAS2 induced antibodies that were able to react with parasite produced antigen.

Rhesus monkeys were immunized with the human clinical dose, 50 μg, of MSP-1_{42} (3D7)/SBAS2 or with MSP-1_{42} (3D7)/alum to access safety and immunogenicity. Each group comprised of eight Rhesus monkeys, which were immunized intramuscularly at 0, 1 month, and 3-month intervals. For both groups no adverse local responses were observed and all biochemical and hematological laboratory tests were normal. Sera were collected 2 weeks following each immunization and antibody titers were analyzed by ELISA with

plates coated with yeast expressed MSP-1_{19}. Antibody titers were reported in ELISA units. For the group in which antigen was formulated with SBAS2, MSP-1 specific antibodies were induced after the 2^{nd} immunization, and peaked at 10,000 ELISA units 2 weeks following this immunization. In this group, the antibody titers declined steadily over the next 7 weeks. Two weeks following the 3^{rd} immunization, antibody titers peaked at a mean of 19,000 ELISA units. Antibody titers induced by immunization with MSP-1_{42} (3D7)/SBAS2 were at least 10 fold greater than those induced by immunization with MSP-1_{42} (3D7)/Alum (Figure 4).

Discussion

The production of an inexpensive, safe and efficacious vaccine for malaria has proven to be difficult. Expression of malaria antigens in heterologous expression systems has been difficult due to the substantial codon bias toward A-T rich sequences. Typically these antigens have either been expressed in insoluble forms or have been expressed poorly within the heterologous expression hosts. We have described the development of a fermentation and purification process for the production of MSP-1_{42} subunit vaccines that are safe and can induce MSP-1 specific antibodies in mice and Rhesus monkeys. These data support the use of MSP-1_{42} (3D7)/SBAS2 in a Phase I clinical trial.

Previous studies have shown that the induction of antibody responses to epitopes on MSP-1_{19} correlated with clinical immunity to malaria. Therefore, properly formed disulfide-dependent conformational epitopes on recombinant MSP-1_{19} or MSP-1_{42} molecules are required for the induction of protective

antibody responses. These *E. coli* expressed recombinant MSP-1_{42} 3D7 and FVO appeared to contain some structurally correct epitopes because they reacted with 8 MSP-1_{19} specific mAbs including functional mAbs classified as growth or invasion inhibitory (mAb 12.10, 12.8) and blocking inhibitory mAbs (mAb 7.5, 2.2, 1E1). Immune sera from mice immunized with the highest dose of MSP-1_{42} (3D7)/SBAS2 were reactive on methanol-fixed parasites by IFA's suggesting that immunization with recombinant MSP-1_{42} (3D7) induced antibodies to native MSP-1. These data support the use of bacterial-expressed MSP-1_{42} (3D7) as an immunogen for the induction of protective antibodies. The MSP-1_{42} (FVO) version has not been characterized to the same extent as the (3D7). We plan to characterize the October 2000 cGMP production lot of the MSP-1_{42} (FVO) for immunogenicity in mice and Aotus monkeys.

Currently, the only malaria vaccine tested in a Phase I trial that has reproducibly protected volunteers at the 50-70% level has been RTS,S/SBAS2 developed by Smithkline Beecham, Rixensart, Belgium (Stoute, 1997). Safety and efficacy data from these studies support the combination of the SBAS2 adjuvant with MSP-1_{42} (3D7) for the induction of protective immune responses.

Key Research Accomplishments

1. Produced 300L of cGMP fermentation paste from *E.coli* Expressed *P. falciparum* (3D7) MSP-1_{42}. (June 1999)
2. Produced approximately 10,000 doses of MSP-1_{42} (3D7) bulk antigen for clinical trials. (June 1999)
3. Produced 600 single dose vaccine vials containing MSP-1_{42} (3D7) for formulation with adjuvant. (June 1999)
4. Produced 1400 single dose vaccine vials containing MSP-1_{42} (3D7) for formulation with adjuvant. (April 2000)
5. Produced approximately 8,000 doses of MSP-1_{42} (3D7) bulk antigen for future trials. (April 2000)
6. Produced 600 single dose vaccine vials containing MSP-1_{42} (3D7) for formulation with adjuvant. (April 2000)
7. Completed characterization of manufacturing process of the cGMP purified MSP-1_{42} (3D7) that meets FDA standards for a product to be used in humans.
8. Documentation of the production and characterization of MSP-1_{42} as written in an IND application for the FDA (July 2000).
9. Completed the purification process development for the alternative allelic-form, MSP-1_{42} (FVO). Developed for studies investigating induction of strain specific immunity in an *Aotus* monkey model.
10. Completed two 300L cGMP Fermentations of *E.coli* expressed *P. falciparum* MSP-1_{42} (FVO). (February 2000, March 2000)
11. Produced approximately 600 doses of MSP-1_{42} (FVO) bulk antigen for clinical trials. (October 2000)

12. Produced 600 single dose vaccine vials containing MSP-1_{42} (FVO) for formulation with adjuvant. (October 2000)
13. Prepared reagents, plasmid constructs, protein products, necessary to further characterization the structural and functional activities of MSP-1_{42}.

Reportable Outcomes

1. Manuscript in Preparation:

Evelina Angov, Barbara Aufiero, Michel Van Handenhove, Christian Ockenhouse, Kent Kester, Doug Walsh, Jana McBride, Gray Heppner, Ripley W. Ballou, Carter Diggs, Jeffrey Lyon, "Process Development and Immunogenicity Studies on a Clinical Grade *E. coli* Expressed *P. falciparum* Merozoite Surface Protein-1_{42} (3D7) Vaccine"

2. Short Report

Angov, E., McBride, J.S., Kaslow, D.C., Ballou, W.R., Diggs, C.L., and J.A.Lyon. 1997. Structural analysis of refolded-recombinant *Plasmodium falciparum* MSP1 C-terminal fragment by using conformation-specific monoclonal antibodies. Nature Biotechnology. Biomolecular Design, Form and Function. Vol. 8. Pg 21.

3. Guest Seminar speaker, October 1999:
   ATCC, Manassa, Virginia
   Topic: Purification and Characterization of rMSP1_{42}, an Erythrocytic Stage Malaria Vaccine Candidate

4. ASTMH Conference, Washington DC November 1999
   Oral Presentation:
   Topic: Process Development for Clinical Grade *Plasmodium falciparum* MSP1_{42} (3D7) Expressed In *E. coli*

5. Poster Presentation, Lorne, Australia
   Meeting: Molecular Approaches to Malaria
   Abstract Title: Characterization of clinical grade *P. falciparum* MSP1_{42} (3D7) expressed in *E. coli*

6. Plans to apply for patent. Will be filling a patent disclosure shortly.

Conclusions

These data support the use of an *E.coli* expressed MSP-1_{42} (3D7) adjuvanted with SBAS2 in a Phase I clinical trial. Recombinant MSP-1_{42} appears to be partially structurally correct because it is immunoreactive with at least 8 MSP-1_{19} specific monoclonal antibodies. Antibody reactivity on disulfide-dependent epitopes in the MSP-1_{19} fragment can predict some level of correct structure in recombinant MSP-1_{42}.

Our safety and immunogenicity studies in Balb/c mice and Rhesus monkeys support the use of this recombinant MSP-1_{42} (3D7) when adjuvanted with SBAS2. In both animal models, animals were able to seroconvert to MSP-1 positive antibodies. The antibody titers that were induced reflected the induction of good MSP-1 specific antibody responses.

These data support the further evaluation of MSP-1_{42} (3D7) as a promising erythrocytic-stage malaria vaccine candidate. Future studies include the characterization of the type of antibody specificities induced (blocking versus processing inhibitory) by vaccination with MSP-1_{42} (3D7)/SBAS2. During the CY 2000 3rd quarter, a Phase I safety and immunogenicity clinical trial was initiated at the WRAIR. In addition, during the CY 2000, additional production lots of the MSP-1_{42} (3D7) were cGMP produced and are being characterized for antigen stability, structural relevance as characterized by monoclonal antibody binding, induction of MSP-1 antibodies, and safety relative to the original vaccine lot.

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Figure Legends

Figure 1:
A). SDS-PAGE analysis of cGMP purified MSP-1_{42} (3D7) for total protein by Coomassie Blue staining and Immunoblotting with MSP-1_{19} specific mAbs under nonreducing conditions. A) Coomassie Blue staining of nonreduced protein at 10 μg. Immunodetection with mAbs 7F1 and 12.10 on 10 μg of Purified MSP-1_{42} (3D7). B) mAb reactivities, Shown is a list of monoclonal antibodies used to measure MSP-1_{19} specific and MSP-1_{33} specific reactivities on cGMP produced MSP-1_{42}.

Figure 2:
ELISA assays to measure Seroconversion in mice.
Antibody titers were reported as the dilution of serum that produced 1 absorbance unit in the ELISA (ELISA units). Balb/C mice immunized with 0.1, 0.3, and 1.0ug's of MSP-1_{42} (3D7)/SBAS2. Antibody titers are reported at a 99% confidence interval.

Figure 3:
ELISA assays to measure Immunogenicity in mice.
Balb/C mice were immunized with 0.1, 0.3, and 1.0ug's of MSP-1_{42} (3D7)/SBAS2. MSP-1 specific antibodies are detected against plates coated with yeast MSP-1_{19}. Antibody titers are reported at a 99% confidence interval. Antibody titers were reported as the dilution of serum that produced 1 absorbance unit in the ELISA (ELISA units).

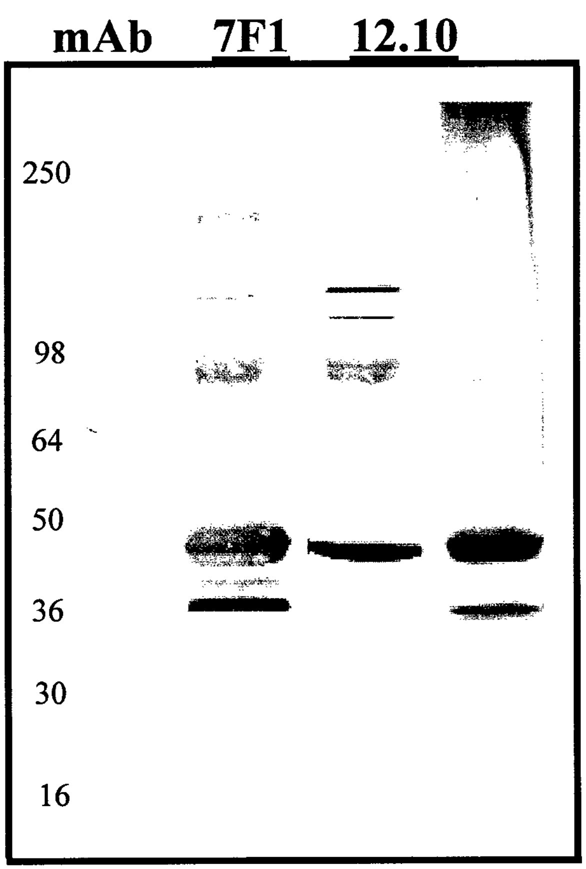
Figure 4:
Immunogenicity in Rhesus. ELISA assay on MSP-1_{42} (3D7) formulated with adjuvants, SBAS2 and Alum. Rhesus monkeys were immunized with 50 ug's of MSP-1_{42} (3D7)/adjuvant. MSP-1 specific antibodies were detected against plates coated with yeast MSP-1_{19}.

Table 1:
Immunogenicity in mice measured by IFA
Sera from mice immunized with 1.0 μg doses of MSP-1_{42} (3D7)/SBAS2 were tested by IFA against methanol fixed parasites. Values are shown as dilutions of mouse sera and their ability to react on parasite lysates.

Figure 1

GMP Purified, *E.coli* expressed MSP-1_{42} from *P. falciparum* (3D7)

A.



B.

mAbs	Specificity	Reactivity
12.10	MSP-1_{19} Domain 1 + 2	++
12.8	MSP-1_{19} Domain 1	++
7.5	MSP-1_{19} Domain 1	++
2.2	MSP-1_{19} Domain 1	++
5.2	MSP-1_{19}	++
1E1	MSP-1_{19}	++
7F1	MSP-1_{33}	++
3H7	MSP-1_{19}	++
3B10	MSP-1_{19}	++

Figure 2

Mouse Potency Study

Seroconversion

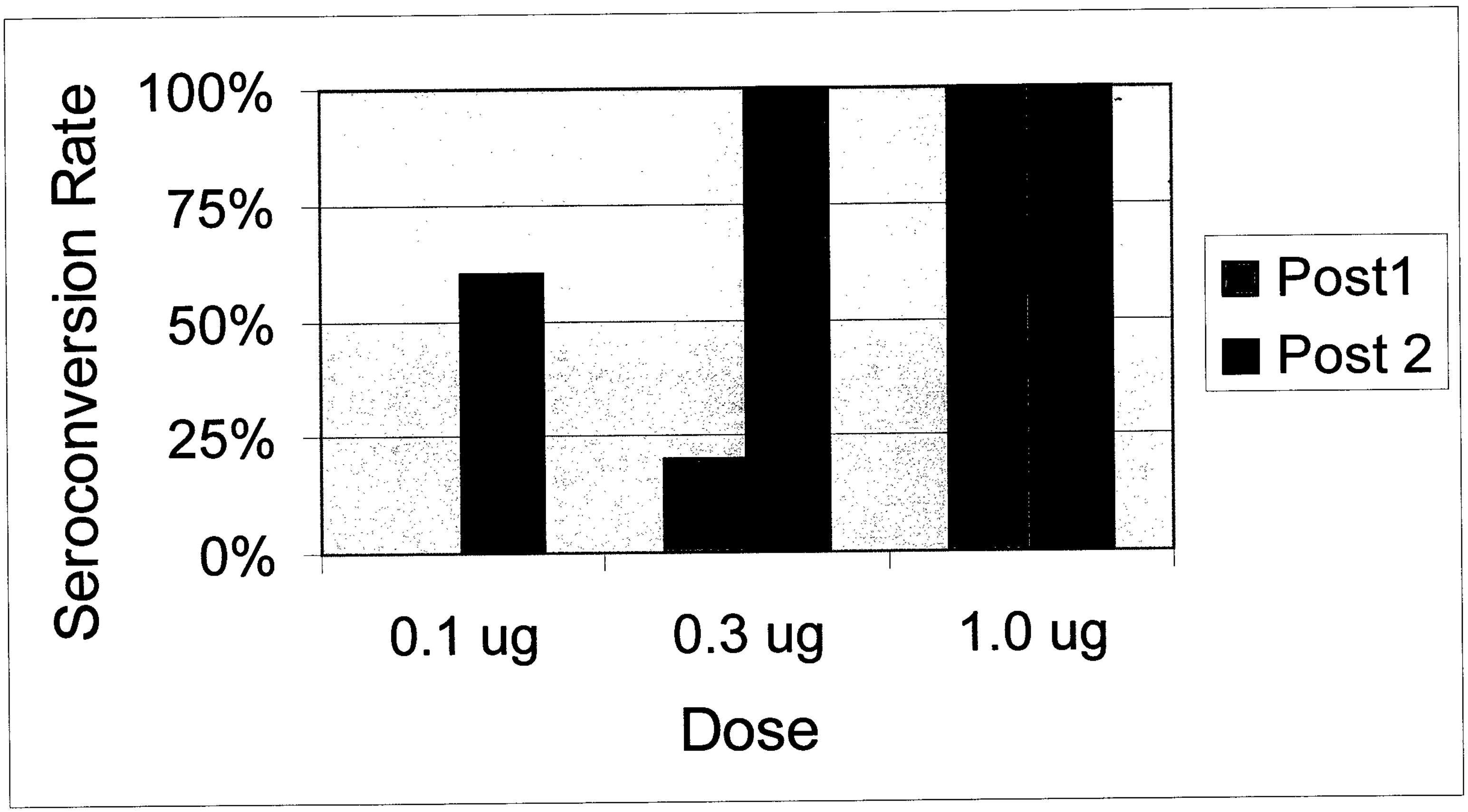
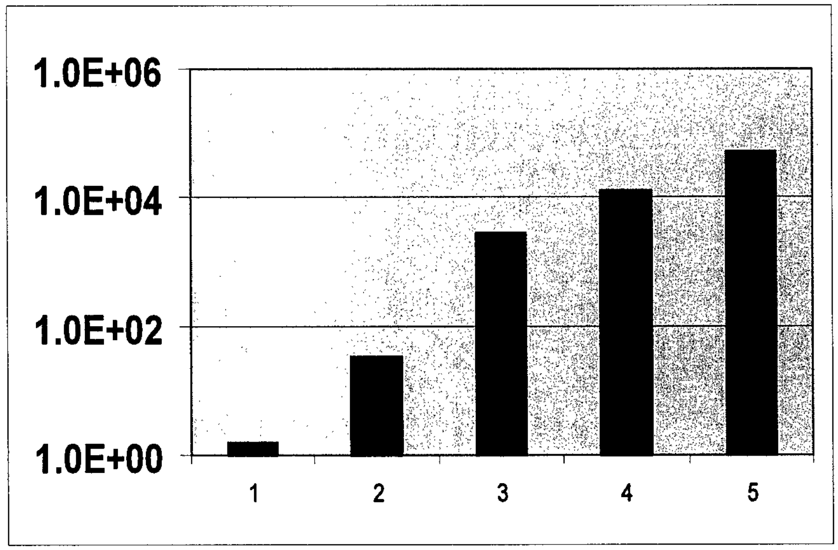


Figure 3

Mouse Immunogenicity

(ELISA)

0.1ug



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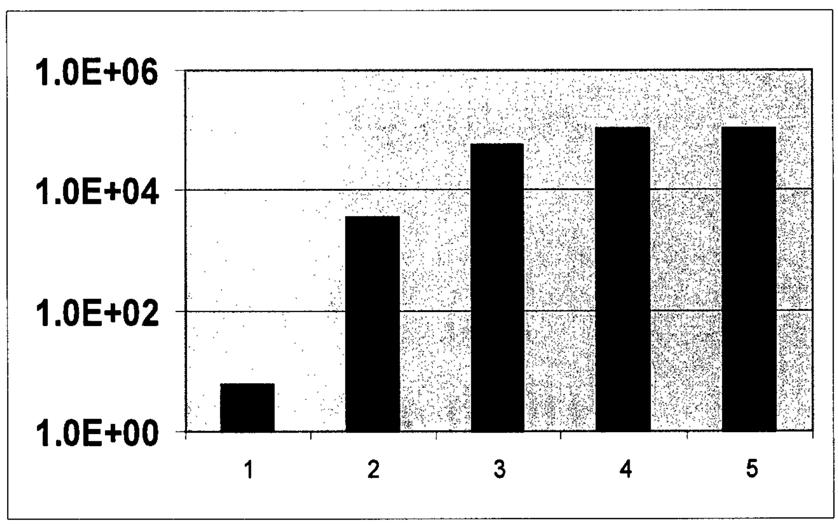

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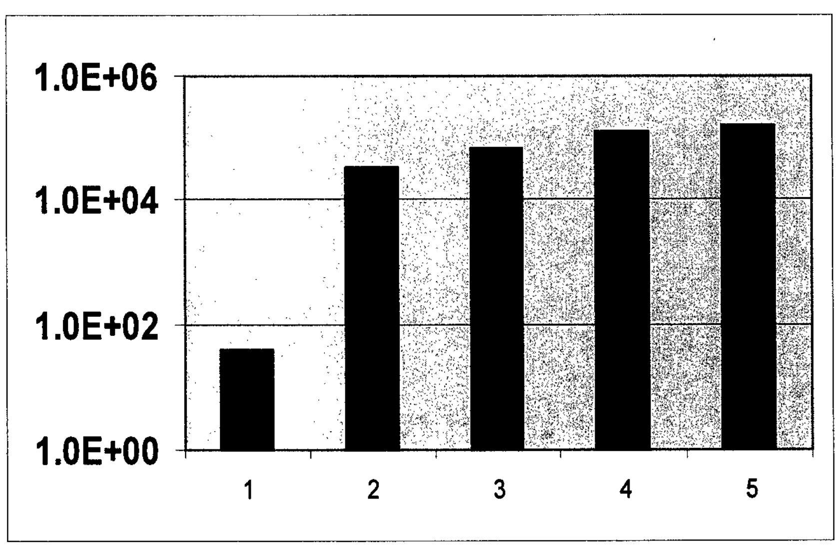

Figure 4

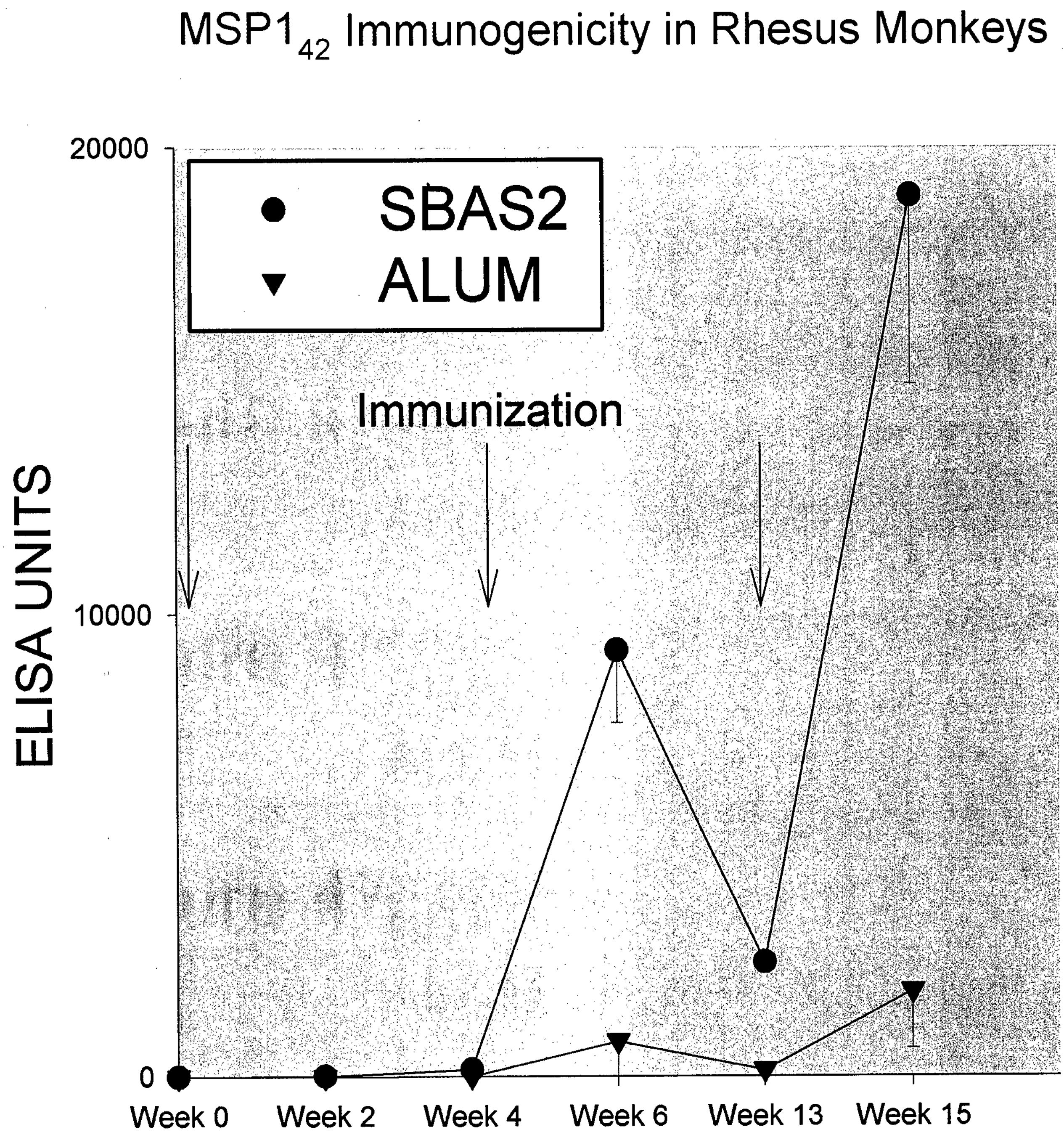
MSP1$_{42}$ Immunogenicity in Rhesus Monkeys
SBAS2
ALUM
Immunization
ELISA UNITS
20000
10000
0
Week 0
Week 2
Week 4
Week 6
Week 13
Week 15

Table 1
Mouse Immunogenicity
by IFA

	Pre-immune	Post 5		
Dilution	1:250	1:1,000	1:3,000	1:10,000
# Seroconverted	0/10	10/10	9/10	7/10

Curriculum Vitae

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EDUCATION

1979-1983 B.A. in Biology and French, Western Maryland College, Westminster, MD.

1983-1986 M.S. in Biochemistry, University of Maryland, College Park, MD. Development of a method to measure the levels of expression of *E. coli* ATPase genes *in vivo* using fusions to lacZ and measuring β-galactosidase activities.

1986-1989 Ph.D. in Biochemistry, University of Maryland, College Park, MD. Major project included the investigation of the translational regulatory features of the polycistronic mRNA encoding *E. coli* ATPase subunits.

POSITIONS HELD

2000-Present 09/30/00 **Microbiologist** (DB-0403)

1995-2000 **Cooperative Agreement Contract** with the U.S. Agency for International Development, (USAID), and the Walter Reed Army Institute of Research, (WRAIR), Walter Reed Army Medical Center, Washington, D.C., to facilitate the development of candidate antigens for malaria vaccine trials. One objective was to characterize available GMP yeast-expressed malaria antigens for identity, purity, and immunoreactivity in order to establish release criteria in support of future human Phase I trials. Additional analysis of some recombinant proteins required that protein-refolding strategies be developed. Conformationally correct structures are probed with a series of reduction-sensitive disulfide-dependent monoclonal antibodies by Western blotting. In addition, alternative expression and purification systems are being investigated, including *E. coli* expression systems developed to increase antigen solubility and correct disulfide bond formation.

1989-1995 **Intramural Research Training Award (IRTA) Fellow** in the Genetics and Biochemistry Branch of the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institutes of Health, Bethesda, MD. Major projects included the isolation and biochemical characterization of the first described thermostable RecA homolog from *Thermus aquaticus* YT-1.

PUBLICATIONS

Angov, E., Aufiero, B., Van Handenhove, M., Ockenhouse, O., Kester, K., Walsh, D., McBride, J., Heppner, G., Ballou, R.W., Diggs, C., Lyon, J.A.,

"Process Development and Immunogenicity Studies on a Clinical Grade *E. coli* Expressed *P. falciparum* Merozoite Surface Protein-1_{42} (3D7) Vaccine". Manuscript in Preparation.

Uthaipibull, C., Aufiero, B.A., Syed, S., Hansen, B., Guevara-Patiño, J., **Angov, E.**, Ling, I.T., Fegeding, K., Morgan, W.D., Ockenhouse, C., Birdsall, B., Feeney, F., Lyon, J.A. & Holder, A.A. Rational design of a malaria vaccine candidate by analysis of inhibitory and blocking monoclonal antibody epitopes on *Plasmodium falciparum* Merozoite Surface Protein 1. (Manuscript submitted to J. Mol. Biol. 2000).

Yu, X., **Angov, E.**, Camerini-Otero, R.D., and E.H. Egelman. 1995. Structural polymorphism of the RecA protein from the thermophilic bacterium *Thermus aquaticus*. Biophys. J. 69:2728-38.

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Angov, E. and W.S.A. Brusilow. 1994. Effects of deletions in the *uncA-uncG* intergenic regions on expression of *uncG*, the gene for the γ subunit of the *Escherichia coli* F_1F_0 ATPase. Biochim. Biophys. Acta. 1183:499-503.

Monticello, R.A., **Angov, E.**, and W.S.A. Brusilow. 1992. Effects of inducing expression of cloned genes for the F_0 proton channel of the *Escherichia coli* F_1F_0 ATPase. J. Bacteriol. 174:3370-3376.

Angov, E., Ng, T.C.N. and W.S.A. Brusilow. 1991. Effect of the δ subunit on assembly and proton permeability of the F_0 proton channel of *Escherichia coli* F_1F_0 ATPase. J. Bacteriol. 173:407-411.

Angov, E. and W.S.A. Brusilow. 1988. Use of *lac* fusions to measure *in vivo* regulation of expression of *Escherichia coli* proton-translocating ATPase (*unc*) genes. J. Bacteriol. 170:459-462.

SHORT REPORT

Angov, E., McBride, J.S., Kaslow, D.C., Ballou, W.R., Diggs, C.L., and J.A. Lyon. 1997. Structural analysis of refolded-recombinant *Plasmodium falciparum* MSP1 C-terminal fragment by using conformation-specific monoclonal antibodies. Nature Biotechnology. Biomolecular Design, Form and Function. Vol. 8. Pg 21.

COURSE LECTURER

May 1996. Foundation for Advanced Education in the Sciences, NIH, Bethesda, MD. BIO-TRAC Course entitled "Antibody Purification and Fragmentation".

July 1997. CATCMB, Catholic University of America, Washington, D.C. Course entitled "Molecular Immunlogy and Immunochemistry" Lecture and Laboratory on PCR technology and Phage Display.

June 1996-ASBMB Abstract:

CHARACTERIZATION OF A RECOMBINANT *PLASMODIUM FALCIPARUM* MSP1 C-TERMINAL FRAGMENT USING CONFORMATION-SPECIFIC MONOCLONAL ANTIBODIES. E. Angov[1], J. S. McBride[2], D. C. Kaslow[3], W.R. Ballou[1], C. L. Diggs[4], and J. A. Lyon[1]. [1]Immunol., WRAIR, Washington, D.C., 20307; [2]Univ. Edinburgh, U.K.; [3]NIAID, NIH, Bethesda, MD 20892; [4]USAID, Washington, D.C., 20523.

The 19kD c-terminal fragment from *Plasmodium. falciparum* MSP1 is a malaria vaccine candidate. Despite its small size, it is relatively complex, containing 12 cysteines that are thought to participate in the formation of 2 EGF-like structures with a total of 6 disulfide bridges. Thus, proper folding and disulfide bond formation may be crucial to generating an effective vaccine from this region. This fragment was expressed in *S. cerevisiae* and purified to homogeneity under GMP. The structure of this product was tested by Western blotting with 5 mAbs known to react with conformation specific epitopes in this region. After separating under non-reducing conditions, the bulk of the antigen did not react with a polyclonal serum nor with monoclonal antibodies, suggesting that the relevant structural epitopes were not formed. However, SDS-PAGE separated, non-reduced antigen that had been reduced in gel with β-mercaptoethanol prior to blotting was reactive with some mAbs. Therefore, this suggests that reduction and refolding of the yeast expressed $MSP1_{19}$ antigen may yield a more relevant malaria vaccine.

January 1997- Short Report-Miami Biotechnology Symposia Abstract:

STRUCTURAL ANALYSIS OF REFOLDED-RECOMBINANT *PLASMODIUM FALCIPARUM* MSP1 C-TERMINAL FRAGMENT BY USING CONFORMATION-SPECIFIC MONOCLONAL ANTIBODIES.

Evelina Angov[1], Jana S. McBride[2], David C. Kaslow[3], W.R. Ballou[1], Carter L. Diggs[4], and Jeffrey A. Lyon[1]. [1]Dept. Immunology, WRAIR, Washington, D.C., 20307; [2]Division of Biological Sciences, Univ. Edinburgh, EH9 3JT, U.K.; [3]NIAID, NIH, Bethesda, MD 20892; [4]USAID, Washington, D.C., 20523.

INTRODUCTION The 19kD c-terminal fragment from *Plasmodium falciparum* merozoite surface antigen, MSP1, is among the leading erythrocytic-stage malaria vaccine candidates(1). Despite its relative small size, it is a complex structure, containing 12 cysteines that are thought to participate in the formation of 2 EGF-like structures with a total of 6 disulfide bridges. MSP1 specific mAbs that are either protective against infection *in vivo* or inhibit parasite growth *in vitro* are specific for reduction sensitive discontinuous epitopes. Thus, proper folding and disulfide bond formation may be crucial to generating an effective vaccine from this region. This fragment was expressed in *S. cerevisiae* (2) and purified to homogeneity under GMP. Protein folding was measured by Western blotting with 5 mAbs known to react with conformation specific epitopes from this region(3).

RESULTS The primary objective of this study was to characterize recombinant products for identity, purity and immunoreactivity. An analytical tool was established which allowed the activity of some reduction-sensitive epitopes to be restored by blocking Western blots in the presence of Tween-20. After SDS-PAGE analysis under non-reducing conditions, the bulk of the antigen did not react with a polyclonal serum nor with monoclonal antibodies, suggesting that the relevant structural epitopes are not formed. However, SDS-PAGE separated, non-reduced antigen that had been reduced 'in gel' with β-mercaptoethanol prior to blotting in the presence of Tween-20 was reactive with some mAbs. Therefore, this data suggests that reduction and refolding of the yeast expressed $MSP1_{19}$ antigen may yield a more relevant malaria vaccine.

Since the data show that the recombinant antigens were highly purified but that a majority of the antigen was misfolded then the heterogeneity observed on Coomassie stained gels was due to one or more incorrect disulfide bonded pairs. Refolding was initiated by reduction of disulfide bonds with either β-mercaptoethanol or dithiothreitol, followed by disulfide bond formation and refolding through dilution and dialysis. Samples were analyzed for correct structure by Western or dot blots. The refolded products formed under specified conditions were represented by either immunoreactive multimers or nonreactive monomers. Carboxymethylation of free sulfhydryl groups on refolded molecules eliminated immunoreactivity suggesting that the aggregated states were formed through some interactions, whether covalent or not, between monomers.

Therefore, the data suggest that the mAbs recognize higher order structures, *i.e.* perhaps epitopes derived from quaternary structures.

CONCLUSION The majority of the yeast-expressed $MSP1_{19}$ vaccine may not be properly folded to elicit an optimal protective immune response. A systematic refolding process may yield higher levels of the correctly folded antigen and therefore may yield a more efficacious vaccine. If the relevant structure on the parasite surface is an assembled form of $MSP1_{19}$, then the refolding process would require that assembly be in the final folding path. However, additional structural analysis is required to determine the nature of the disulfide bonding patterns and whether $MSP1_{19}$ monomers must be assembled into higher order structures.

REFERENCES

1. Diggs, C.L., Ballou, W.R., and L.H. Miller. 1993. The major merozoite surface protein as malaria vaccine target. Parasitol. Today. **9**:300-302.
2. Kumar, S., Yadava, A., Keister, D.B., Tian, J.H., Ohl, M., Perdue-Greenfield, K.A., Miller, L.H. and D.C. Kaslow. 1995. Immunogenicity and *in vivo* efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in *Aotus* monkeys. Mol. Med. **1**:325-332.
3. McBride, J.S. and H.-G Heidrich. 1987. Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites form an antigenic complex. Mol. Biochem. Parasitol. **23**:71-84.

November 1999-ASTMH Abstract:

PROCESS DEVELOPMENT FOR CLINICAL GRADE *PLASMODIUM FALCIPARUM* MSP1_{42} (3D7) EXPRESSED IN *E. COLI.*

The merozoite surface protein-1 (MSP1) of *P. falciparum* is a leading erythrocytic-stage vaccine candidate. It is a 195kDa protein that is proteolytically processed to several fragments, and may play a role in binding and/or invasion of erythrocytes by merozoites. In conjunction with erythrocyte invasion, the most C-terminal fragment (MSP1_{42}) undergoes secondary processing giving a 33kDa and a 19kDa fragment (MSP1_{19}). Although the latter is a target for parasite inhibitory monoclonal antibodies and protective immune responses, it appears to lack T-helper epitopes. Since antibody is most likely the effector mechanism induced by MSP1_{19}, it is important to insure that recombinant vaccines based upon this antigen be folded correctly and contain T-helper epitopes that will enhance induction of humoral responses. To fulfill these objectives we developed recombinant MSP1_{42} molecules that were structurally correct and developed fermentation and purification processes that could advance this product into human clinical trials. Bacterial expression of MSP1_{42} from the pET T7 driven promoter-expression system results in soluble MSP1_{42} that is immunoreactive with several functional MSP1/19\-specific mAbs, including mAbs 12.10, 12.8. 7.5, 7.2, and 1E1. Purification over three chromatographic steps that include nickel affinity chromatography, anion exchange on a Q-substituted resin and cation exchange on a CM-substituted resin, yields protein that is 95% pure and exceeds FDA endotoxin standards. The final lyophilized product is stable. Mice seroconverted following immunization with recombinant MSP1_{42}. Safety and immunogenicity testing has been initiated in Rhesus monkeys.

February, 2000 Lorne, Australia Abstract:

Characterization of clinical grade *P. falciparum* MSP1$_{42}$ (3D7) expressed in *E. coli.*

E Angov, B Aufiero, C Ockenhouse, J McBride[1], K Kester, D Walsh, S Pichyangkul, M Van Handenhove[2], WR Ballou, C Diggs[3], JA Lyon

WRAIR, Silver Spring, MD 20910 U.S.A., [1]University of Edinburgh, Scotland, [2]SmithKline Beecham Biologicals, Belgium, [3]U.S.A.I.D., Washington, D.C., 20523

The merozoite surface protein-1 (MSP1) of *P. falciparum* is a leading erythrocytic-stage vaccine candidate. It is a 195kDa protein that is processed to several fragments, and may play a role in binding and/or invasion of erythrocytes by merozoites. In conjunction with erythrocyte invasion, the most distal C-terminal fragment (MSP1$_{42}$) undergoes secondary processing giving a 33kDa and a 19kDa fragment (MSP1$_{19}$). Although the latter is a target for parasite inhibitory mAbs and protective immune responses, it appears to lack T-helper epitopes. Since antibody is most likely the effector mechanism induced by MSP1$_{19}$, it is important to insure that recombinant vaccines based upon this antigen be folded correctly and contain T-helper epitopes that will enhance induction of humoral responses. To fulfill these objectives, we developed recombinant MSP1$_{42}$ molecules that were structurally correct and developed fermentation and purification processes that could advance this product into human clinical trials. Bacterial expression of MSP1$_{42}$ from the pET T7 driven promoter-expression system produced soluble MSP1$_{42}$, which reacted with several functional MSP1$_{19}$-specific mAbs, including mAbs 12.10, 12.8. 7.5, 7.2, and 1E1. Purification over three chromatographic steps that included Ni^{+2} affinity chromatography, anion exchange on a Q-substituted resin and cation exchange on a CM-substituted resin yielded 95% pure protein that exceeded FDA endotoxin standards. The final lyophilized product was stable. Mice seroconverted following immunization with recombinant MSP1$_{42}$ adjuvanted with SBAS2. Safety and immunogenicity testing in Rhesus monkeys showed that MSP1$_{42}$ in combination with SBAS2 was safe and induced MSP1$_{42}$ specific antibody responses.